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Over-expression of the HER-2/neu oncogene in breast cancers is associated with aggressive tumor formation, shorter relapse times and higher chemo-resistance. In an effort to transcriptionally downregulate HER-2/neu expression, we have targeted the gene promoter's TATA box with DNA minor groove-binding hairpin polyamides. The DNA-binding specificity and affinity of a 6 bp-binding hairpin polyamide for the Her-2/neu TATA box was determined by the combinatorial method REPSA and confirmed by DNase I footprinting. EMSA studies showed that the hairpin polyamide could inhibit TBP binding to the HER-2/neu TATA box at nanomolar concentrations. A preliminary analysis using a promoter reporter construct in SKBR-3 breast cancer cells revealed that this polyamide molecule could indeed interfere with transcription from the Her-2/neu promoter. The AP-2 binding site in the Her-2/neu promoter was also targeted with a recombinant repressor protein, which showed a promising result.

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INTRODUCTION

The Her-2/neu oncogene is over-expressed in about 30 % of female breast cancers and is an adverse prognostic indicator of aggressive malignancy characterized by chemotherapeutic resistance and shorter relapse times (1). The purpose of this project is to down-regulate transcription of the Her-2/neu oncogene using sequence-specific DNA binding molecules that interfere with transcription factor binding to the gene promoter. Specific targets under study are the TATA box and the AP-2 binding site of the Her-2/neu promoter. Down-regulating the expression of this oncogene in over-expressing breast cancer cells should reduce the malignant phenotype of these cells and sensitize them to chemotherapeutic agents. Successful application of this methodology in cell culture would increase the scope of this project for therapeutic development in animal experiments and clinical trials.

BODY

A. Specific aims for the year of report.

1. Identification of the best binding hairpin polyamide species that bind with the highest specificity and affinity to the target sequences of the Her-2/neu promoter.
2. Effect of this binding on the normal recognition by promoter binding factors.

B. Studies and results:

Identification of the hairpin polyamides that bind with the highest affinity and specificity to the promoter elements of the Her-2/neu gene.

The most important transcriptional target for down-regulating Her-2/neu gene expression would be the core promoter consisting of the TATA box and the start site of transcription. In order to prevent/minimize non-specific interactions with the TATA box regions of other genes, two nucleotides (-AG-) flanking the 5′ end of the Her-2/neu TATA box were included as part of the recognition sequence for the hairpin polyamides. A promoter database search was carried out to analyze the frequency of this target sequence (-AGTATA-). Sequence analysis of the TATA box regions in the Eukaryotic Promoter Database (EPD) revealed that this -AG- flanking TATA sequence was found in less than

4% of the human type II gene promoters, which include the promoters of c-myc, collagen I, α-fetoprotein, keratin I, opsin, etc.

The hairpin polyamides under study were synthesized at Genesoft Inc., San Francisco, CA by solid phase synthetic methods (2). The hairpin polyamides that could bind to the sequence AGTATA, as determined by the side-by-side pairing rules for these molecules (3), were analyzed for binding specificity using the combinatorial method- Restriction Endonuclease Protection, Selection and Amplification (REPSA) developed in our lab (4). The schematic of REPSA is shown in Figure 1A and 1B. Chemical structures of two hairpin polyamides ImPyPyPy-γ-ImPyPyPy-β-Dp and ImPyPyPy-γ-PyPyPyPy-β-Dp along with the schematics of their expected consensus DNA recognition sites are shown in Figure 1C. Emergence of preferred binding sequences for the hairpin polyamides in the successive rounds of REPSA is shown in Figure 2A. The binding affinities of the REPSA selected sequences were determined by a Restriction Endonuclease Protection assay (REPA). A representative REPA assay on four REPSA selected sequences for the polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp is shown in Figure 2B. Figure 2C shows the binding affinity (% A) of some REPSA selected sequences determined by REPA for the two hairpin polyamides. DNase I footprinting was carried out to verify exact binding sites in the REPSA selected clones. Dissociation constants were determined using a non-linear regression analysis using a single site binding hyperbolic equation $I=I_{max} \times [hp]/[k_d=hp]$, where I is cleavage inhibition, I_{max} is maximum extent of cleavage inhibition observed, [hp] is the hairpin polyamide concentration and k_d is the dissociation constant. Representative footprints for the sequences AGTATA and AGTACA are shown in Figure 3. The k_d values for a few sequences with high affinities are shown in the table below, along with those with a single base pair mis-match (in bold).

binding site	Kd (nM)
ImPyPyPy-γ-PyPyPyPy-β-Dp	
5′-TGTTTT-3′	38
5′-AGTATA-3′	43
5′-TGTTAA-3′	47
5′-**AGTACA**-3′	**216**

ImPyPyPy-γ-ImPyPyPy-β-Dp	
5′-AGTACT-3′	36
5′-AGTACA-3′	54
5′-TGTTCT-3′	42
5′-**AGTATA**-3′	**190**

The data shows that the polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp has high binding affinity for AGTATA along with TGTTTT and TGTTAA but not AGTACA. On the other hand, the polyamide ImPyPyPy-γ-ImPyPyPy-β-Dp has high affinity for AGTACA and very low affinity for AGTATA.

Overall, DNA binding analysis using REPSA and DNase I footprinting showed that the hairpin polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp has a high binding specificity for the sequence –AGTATA-, which fits with our target of the Her-2/neu TATA box. Also, the binding affinity for this sequence is comparable to that of TBP for its consensus TATA sites (5).

N-methyl pyrrole pairings (Py-Py) in a hairpin polyamide recognize either A-T or T-A base pairs in a degenerate manner, but with high affinity. The N-methylimidazole and N-methylpyrrole pairings do not show this degeneracy, that is, Im-Py pairing shows specificity for G-C and Py-Im pairing shows specificity for C-G. However, larger polyamides that recognize G-C rich sequences greater than 6 bp have lower levels of specificity and affinity for those sequences. Due to this reason, we have not yet been able to identify polyamide species that would have a specific interaction with the G-C rich AP-2 binding site of the Her-2/promoter (-GCTGCAGGC-). Smaller polyamides that target a smaller region of the AP-2 site were also not considered because these sites were found to have sequence similarity with the sites for other transcription factors like Sp1. This stringency for sequence requirement is necessary to avoid the potential pitfalls of present day chemotherapeutics. However, with the development of new amide groups which show improved recognition over longer stretches of G-C rich DNA (6, 7), we are hopeful to generate polyamides that specifically recognize the AP-2 site and other sites that could be important transcriptional targets in the Her-2/neu promoter.

Effect of hairpin polyamide binding on the normal recognition by promoter binding elements.

The TATA binding protein (TBP) as part of the holo-TFIID complex plays a critical role in the transcription of class II genes. TBP forms minor groove contacts with an 8 bp stretch of DNA through its positively charged, saddle-shaped convex surface. Hairpin polyamides also bind in the minor groove of DNA. Hence the molecule ImPyPyPy-γ-PyPyPyPy-β-Dp, which has high affinity for the sequence AGTATA, would have the potential to replace or compete with TBP for binding to the TATA box of Her-2/neu. The ability of this hairpin polyamide to interfere with TBP binding to the TATA box of the Her-2/neu promoter was examined using an electrophoretic mobility shift assay. A 30 bp TBP binding probe consisting of the Her-2/neu promoter DNA from –10 to –39 relative to the transcription start site was chemically synthesized and 3′-labeled with ^{32}P. This probe (EMTAT-1) was incubated with increasing concentrations of the hairpin polyamide in a buffer containing 10 mM Tris-Cl (pH 7.5), 0.05 mM EDTA, 0.5 mM DTT, 0.01% NP-40, 10 % glycerol and 5 mM $MgCl_2$ for 30 min at 30 ^{0}C. Recombinant human TBP (purified following the procedure described in ref. 8) was added to the DNA and incubated for an additional 30 min. The samples were then electrophoresed on 6 % TBE gels and visualized on a phosphor imager. The polyamide completely inhibits TBP binding at a concentration of 200 nM. This data clearly indicates that the hairpin polyamide could effectively inhibit TBP binding to the TATA box.

Reporter assay for preliminary analysis of Her-2/neu down-regulation:

To determine whether inhibition of TBP binding *in vitro* translates into down-regulation of Her-2/neu promoter activity *in vivo*, a luciferase reporter assay was carried out. A Her-2/neu promoter reporter (pNeulit) which contains the promoter sequence from -500 to +30 in a pGL2 vector was transfected into the SKBR-3 breast cancer cells. The cells were treated with the hairpin polyamides ImPyPyPy-γ-PyPyPyPy-β-Dp and ImPyPyPy-γ-ImPyPyPy-β-Dp. The results show that the polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp could inhibit luciferase activity significantly at 200 nM, where as the polyamide ImPyPyPy-γ-ImPyPyPy-β-Dp which has a one base pair difference in binding, could not appreciably do so. This is a clear indication that inhibition of TBP binding to the TATA

box may be responsible for down-regulation of Her-2/neu promoter activity by ImPyPyPy-γ-PyPyPyPy-β-Dp. In order to analyze if the AP-2 binding site could be an appropriate target for down-regulating Her-2/neu expression, a pCMV plasmid expressing a recombinant protein, KAP-2 (consisting of a KRAB repressor motif fused to the AP-2 protein), was constructed. The KRAB (krüppel-associated box) motif is a potent repressor of gene transcription, which acts either by histone deacetylase activity and/or co-repressor recruitment (9, 10). This plasmid was co-transfected into the cells along with the pNeulit reporter and luciferase activity was measured after 72 h. The result shows that this repressor protein has a dominant negative effect on the Her-2/neu promoter activity. This not only suggests that the AP-2 binding site is an important target for down-regulating Her-2/neu expression, but also that the KAP-2 repressor may be a promising gene therapeutic with potential for breast cancer gene therapy.

In light of this surprisingly promising result with the KAP-2, we wish to include this repressor protein along with the hairpin polyamides in our original protocol for down-regulating the expression of Her-2/neu oncogene. This does not involve any changes in the original statement of work. An additional gene therapeutic approach would be used in combination with the hairpin polyamides to achieve higher levels of Her-2/neu down-regulation.

KEY RESEARCH ACCOMPLISHMENTS

1. Using the REPSA methodology, we have determined the preferred DNA-binding sites for hairpin polyamides that hold promise for use in down-regulating Her-2/neu expression. This analysis not only identified the best hairpin polyamide for down-regulating Her-2/neu expression, but also identified binding sites for the molecule that could be helpful in understanding other pleiotropic effects associated with its *in vivo* use.
2. EMSA analysis showed that the hairpin polyamide, ImPyPyPy-γ-PyPyPyPy-β-Dp could inhibit binding of TBP to the Her-2/neu TATA box.
3. The luciferase reporter assay provided evidence that inhibition of TBP interaction leads to down-regulation of Her-2/neu promoter activity.

REPORTABLE OUTCOMES

A manuscript entitled "A Combinatorial Determination of Sequence Specificity for Nanomolar DNA-binding Hairpin Polyamides" is being readied for publication.
Authors: Y.N. Vashisht Gopal and Michael W. Van Dyke.

Down-regulation of Her-2/neu gene expression by hairpin polyamides is another reportable outcome in the form of a manuscript, which will be prepared for publication in the near future.

CONCLUSIONS

Determination of DNA-binding affinity and specificity following the methodology detailed above is an important exercise to select for DNA-binding therapeutics that would show the highest degree of preference for specified DNA sequences. Such analysis could also provide the basis to explain varied pleiotropic effects associated with non-targeted or unwanted interactions. The displacement of an important transcription apparatus like the TFIID from a specified sequence by a small minor groove-binding molecule is a significant effect with therapeutic implications. Down-regulation of the Her-2/neu promoter activity using a hairpin polyamide is proof that sequence-specific interaction by small molecules, whose binding affinity is similar to transcription factors, may have the potential to regulate gene expression for therapeutic purposes. The transient transfection study with the recombinant KAP-2 repressor protein suggested that the AP-2 binding site is an important target for down-regulating Her-2/neu expression and also that the KAP-2 repressor may have potential as a gene therapeutic. Polyamides that effectively target the AP-2 binding site would also be studied.

Recommended changes/additions to better address the research topic:

We do not wish to make any changes to the original statement of work, but would like to include an additional gene therapeutic protocol to better achieve the goals of our research project. During the second and third years of the grant period, we would like to explore the effect of different repressor proteins that bind at the AP-2 site. Since multiple KRAB motifs have been shown to have additive repressor effects, The KRAB repressor motif

would be fused singly or in multiples to the N-terminal and/or C-terminal end of AP-2 or only the DNA binding domain of AP-2. Luciferase reporter assays and proliferation assays would be carried out to determine the most effective repressor proteins that down-regulate Her-2/neu expression and cause cytostasis/apoptosis. These will be tested in combination with ImPyPyPy-γ-PyPyPyPy-β-Dp to determine if inhibition of TBP binding by hairpin polyamide and inhibition of AP-2 binding by a dominant negative repressor protein would have an additive effect or a more potent synergistic effect on Her-2/neu down-regulation. A synergistic effect of Her-2/neu down-regulation and chemotherapeutic response has been shown in earlier studies (11, 12). Such phenomena would also be explored with the hairpin polyamides, repressor proteins and chemotherapeutics like doxorubicin and paclitaxel.

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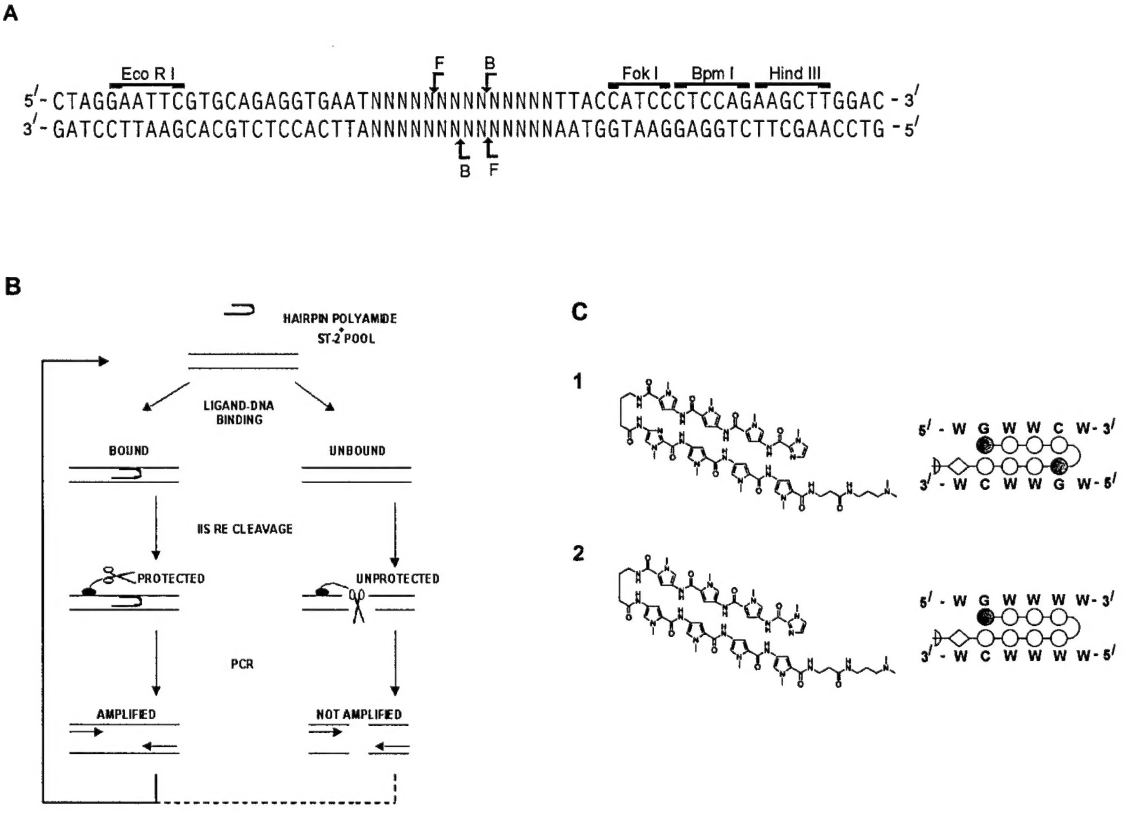
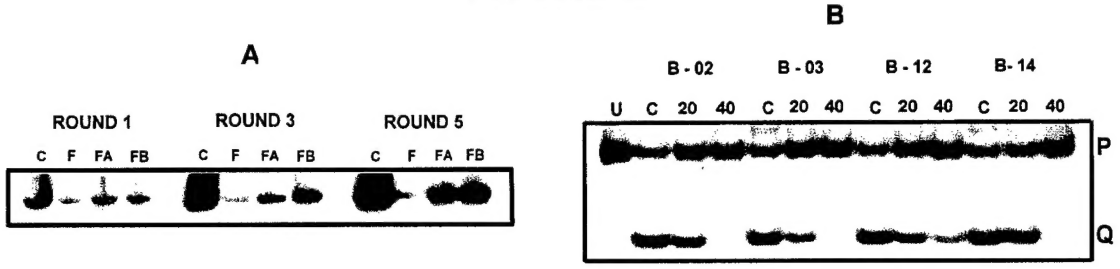
FIGURE 1



(A) REPSA is carried out using a selection template ST2, which is a 63 bp DNA segment, with a 14 base pair random sequence region $(N)_{14}$. The random region is flanked by *Fok I* and *Bpm I* binding sites indicated by brackets over their respective recognition sites. These type IIS restriction enzymes bind to their recognition sites, but cleave within the randon region. The regions within which *Fok I* and *Bpm I* cleave the DNA are represented by F and B. ***(B)*** Flowchart of the combinatorial method REPSA showing the three steps involved- the ligand binding step, in which the hairpin polyamide is allowed to bind to the ST-2 template; the IISRE cleavage step, in which the enzyme binds sequence-specifically to the template DNA, but cleaves it non sequence-specifically in the random region; and amplification step in which the cleavage protected DNA is amplified and fed into the next round of REPSA to continue the combinatorial selection cycle. The hairpin polyamide is represented by a hairpin structure and the IISRE by an oval bearing a scissors. During each round of the REPSA selection, ligand bound sequences are amplified and unbound sequences are digested. After 5 rounds of selection by *Fok I* and one round of selection by *Bpm I*, highly enriched hairpin polyamide binding sequences arise in the N_{14} region, which are subcloned into pUC19 plasmid and transfected into XL1-blue cells. About 60 clones bearing the enriched templates are selected randomly and the N_{14} region sequenced to determine the hairpin polyamide binding sequences. ***(C)*** Chemical structures of the hairpin polyamides ImPyPyPy-γ-ImPyPyPy-β-Dp (1) and ImPyPyPy-γ-PyPyPyPy-β-Dp (2) are shown on the left and schematics of their DNA consensus recognition on the right. A pairing of Im (grey circle) with a Py (white circle) recognizes a G·C base pair and vice versa, while a Py-Py pairing recognizes A·T or T·A (complementary A·T and T·A are represented as W·W).

FIGURE 2



C

ImPyPyPy-γ-PyPyPyPy-β-Dp

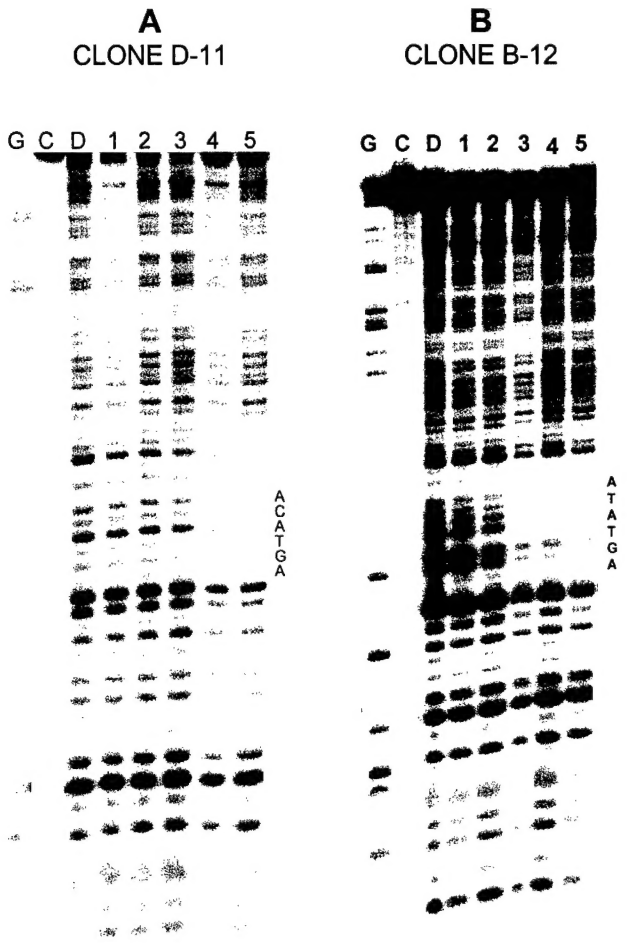
clone	sequence	%A
B-01	ATAGGTACCA**TGTT** **TTAC**	+++
B-02	CC**TGTTTTT**ATAGC TTAC	+++
B-03	TAAT**TGTTTT**AGTA TTAC	+++
B-04	**TTGTTTTTT**GCACA TTAC	+++
B-05	**TGTTTA**TTCTAATT TTAC	++
B-06	TAACC**TGTTAA**AAC TTAC	+++
B-07	GTAT**TGTTAA**CATC TTAC	+++
B-08	GGCT**TTAACA**TATA TTAC	+++
B-09	ATAT**TGTTA**TTCAT TTAC	+++
B-10	**ATGTAAAGTGTTTA** TTAC	+++
B-11	AATT**TGATTTTTTT** TTAC	++
B-12	TGTTAC**AGTATA**GT TTAC	+++
B-13	TCACGA**TGTAAA**GA TTAC	+
B-14	TGT**AGTATA**GGGAA TTAC	+++
B-15	ATTAC**ATTACT**AGC TTAC	++
B-16	**GAGTTTA**TCTTAGT TTAC	++
B-17	CT**TAACT**AGCCTTG TTAC	++
B-18	ATTTTTTGG**AGATT** TTAC	+
B-19	**TTTTCT**TGACTATC TTAC	+
B-20	TAACTAC**AGTTTTT** TTAC	+
B-21	GT**TCATAA**GCTTGG TTAC	++
B-22	TGCAATGG**TTTACT** TTAC	+
B-23	CCTATCC**AGTAAAT** TTAC	+
B-24	**ATTATCAAAATTGA** TTAC	++
B-25	TTTATTTT**TAAAGA** TTAC	+
B-26	TGTCTCGTTT**TATC** TTAC	++
B-27	CCCA**TCTTTA**ACAA TTAC	+

ImPyPyPy-γ-ImPyPyPy-β-Dp

clone	sequence	%A
D-01	AGTTTCT**AGTTCA**T TTAC	+++
D-02	AGGTC**AAGTTCA**AT TTAC	+++
D-03	TAAACTC**AGTTCAA** TTAC	+++
D-04	TTTTGTAA**ACTTGA** TTAC	++
D-05	TATTAATTT**AGTTC** TTAC	++
D-06	TGAGGTAGA**AGTTC** TTAC	++
D-07	CACTCATT**TGTTCT** TTAC	+++
D-08	ATTT**TGTTCT**TCAA TTAC	+++
D-09	GGTTG**AGTACT**CAG TTAC	+++
D-10	ACCG**AGTACT**CTGA TTAC	+++
D-11	GATCTTA**AGTACA**T TTAC	+++
D-12	TATCCTA**TGTTCA**T TTAC	++
D-13	AGT**TGTTCA**TTTTA TTAC	++
D-14	GAAATT**TGTTCA**TT TTAC	++
D-15	TACAG**TGAACA**ACT TTAC	+
D-16	CGTCAAT**TGTACT**A TTAC	+++
D-17	**GTACT**TACACCAAA TTAC	++
D-18	TTCATT**TGTACT**TT TTAC	+++
D-19	TTTATCTA**TGTACT** TTAC	++
D-20	GAACATTGT**TGTAC** TTAC	++
D-21	TGGTCTGGA**TGTAC** TTAC	++
D-22	ATTC**TCAAGT**AACT TTAC	+
D-23	**GTCTAGT**ATATCTA TTAC	+
D-24	TGTGC**TGAACT**CAC TTAC	++
D-25	TGTGC**TGAACT**CAC TTAC	++
D-26	**TGAACT**TAATCTGT TTAC	+
D-27	**TTGAACA**AGGTGTG TTAC	+

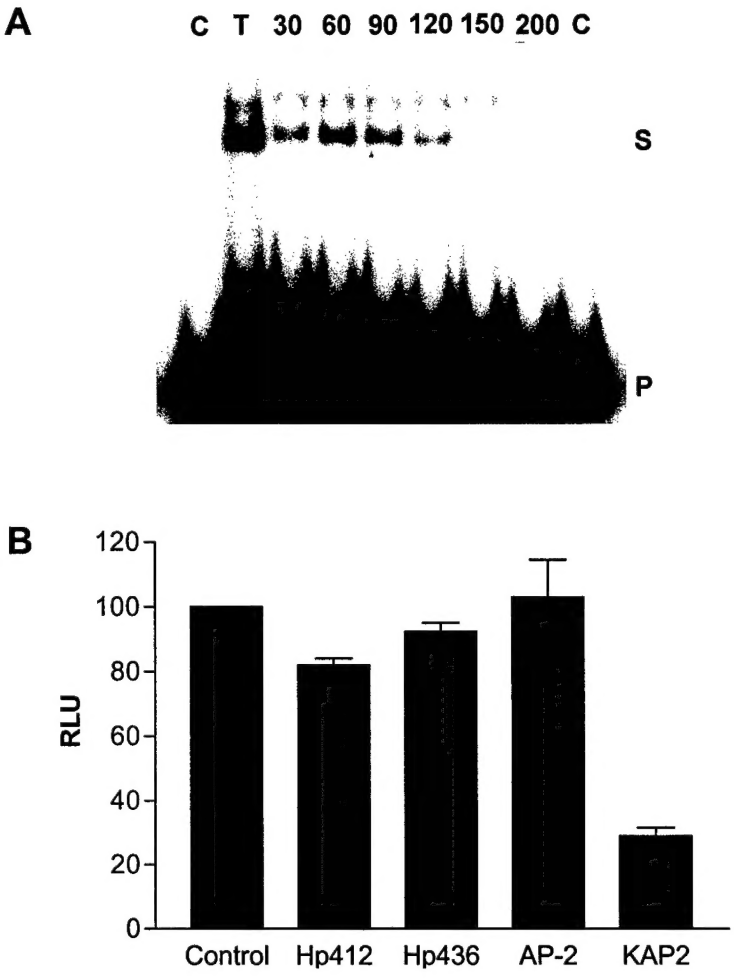
(A) Emergence of a IISRE cleavage resistant population after five rounds of REPSA. The figure shows ^{32}P-labeled ST2 DNA from the first, third and fifth rounds of REPSA, resolved in a TBE-PAGE gel. ST-2 DNA (**C**) was digested with *Fok I* in the absence (**F**) or presence of the hairpin polyamides ImPyPyPy-γ-ImPyPyPy-β-Dp (**FA**) and ImPyPyPy-γ-PyPyPyPy-β-Dp (**FB**). The results show that in the first round of REPSA, over 95% of the ST-2 DNA was cleaved by *Fok I* in the absence of the hairpin polyamides, while in the presence of the hairpin polyamides, ~ 85% was cleaved. Subsequently in the REPSA selection, DNA incubated with the hairpin polyamides becomes more *Fok I* resistant, as seen in the **FA** and **FB** lanes of the third and fifth rounds of REPSA. This *Fok I* resistance indicates the emergence of a significant hairpin polyamide binding population of ST-2 after the fifth round. ***(B)*** Restriction endonuclease protection assay (REPA) to determine *Fok I* cleavage protection conferred by the hairpin polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp on the REPSA-selected clones B-02, B-03, B-12 and B-14. ^{32}P-labeled DNA (**U**) from the REPSA clones was subjected to *Fok I* digestion in the absence (**C**) or presence of 20 nM (**20**) and 40 nM (**40**) ImPyPyPy-γ-PyPyPyPy-β-Dp. '**Q**' indicates *Fok I*-cleaved DNA and '**P**' indicates the DNA protected from cleavage. ***(C)*** REPSA selected sequence clones containing the consensus sites for the hairpin polyamides ImPyPyPy-γ-PyPyPyPy-β-Dp and ImPyPyPy-γ-ImPyPyPy-β-Dp in the 14 base pair random region. Four bases flanking the 3′ end of the random region are also seen. The hairpin polyamide binding consensus sites in the forward or reverse orientation are shown in bold; overlapping sites or a second site are underlined. Clones showing greater than 30%, 50% and 70% cleavage protection at 40 mM hairpin polyamide concentration in the *Fok I* protection assay are indicated by +, ++ and +++ respectively at the right of each sequence (%A).

FIGURE 3



DNase I footprinting for identification of hairpin polyamide binding sites on the REPSA-selected DNA clones D-11 and B-12. ^{32}P-labeled PCR-amplified DNA from the clones (lane **C**) was incubated with 10, 20, 40, 60 and 80 nM (lanes **1**, **2**, **3**, **4**, **5**) of ImPyPyPy-γ-ImPyPyPy-β-Dp ***(A)*** and ImPyPyPy-γ-PyPyPyPy-β-Dp ***(B)*** for 1 h. at 37 ^{0}C. The DNA was then subjected to DNase I cleavage (lane **D**) at RT for 30 s. Products of G-specific chemical sequencing reaction (lane **G**) served as electrophoretic markers. The DNase I digestion products were resolved on a high resolution denaturing PAGE and visualized by autoradiography. The regions of the footprints corresponding to the hairpin polyamide binding sites (AGTACA and AGTATA) are represented at the right of the footprint.

FIGURE 4



(A) Electrophoretic mobility shift assay on the EMTAT-1 probe (lane C) showing the shift induced in the DNA by TBP (lane T). Increasing concentrations of the hairpin polyamide ImPyPyPy-γ-PyPyPyPy-β-Dp in nM concentration are indicated over the respective lanes. **P** and **S** on the right of the gel indicate the free probe and the TBP shifted probe. The hairpin polyamide was able to completely inhibit TBP binding at a concentration of 200 nM. **(B)** A Luciferase reporter assay carried out in SkBr-3 cells. A Her-2/neu promoter reporter plasmid (pNeulit) was transfected at a concentration of 1μg into 50,000 cells using the Fugene 6 reagent. After 6 h. incubation, the hairpin polyamides (200 nM) were added and the cells incubated for 72 h. Similarly, 1μg of eukaryotic expression plasmids expressing AP-2 (pCMV-AP2) and KAP-2 (pCMV-KAP2) were co-transfected into the cells along with the reporter and the cells were incubated for 72 h. A β-galactosidase control vector (pSV-βgal) was used as a transfection control. After incubation, the cells were lysed and the luciferase activity was measured using a promega luciferase assay system. The data was expressed as relative light units in a mean of triplicates histogram. On the x-axis, Control shows the luciferase activity in cells transfected with pCMV empty vector, AP-2 and KAP-2 show the luciferase activities in cells transfected with pCMV-AP2 and pCMV-KAP2. Hp436 and Hp412 show the luciferase activity in cells treated with the polyamides ImPyPyPy-γ-PyPyPyPy-β-Dp and ImPyPyPy-γ-ImPyPyPy-β-Dp.